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KONJUGAT MELLAN MODIFIERAT SUPERANTIGEN OCH EN MÅLSÖKANDE FÖRENING SAMT ANVÄNDNING AV KONJUGATEN.

Superantigen är en grupp av föreningar, främst proteiner av viralt eller bakteriellt ursprung, som har förmåga att samtidigt binda till MHC klass II antigen på mammalieceller och till Vβ-kedjan hos T-cell receptorn (TCR) med följd att T-lymfocyter aktiveras för lys av den MHC klass II bärande cellen. På grund av en moderat grad av polymorfi hos den bindande delen i Vβ-kedjan kommer en relativt stor andel T-lymfocyter att aktiveras (jämfört med den aktivering som sker vid normal "antigen-processing") vid kontakt med ett superantigen.

Från början sattes begreppet superantigen främst i samband med olika stafylokockenterotoxiner (SEA, SEB, SEC_1, SEC_2, SED och SEE). När intresset väl väcktes upptäcktes ytterligare superantigen. Exempel är "toxic shock syndrome toxin 1" (TSST-1), "exfoliating toxins" (ExFt) som är associerade med "scalded skin syndrome", "Streptococcal pyrogenic exotoxin" A, B och C (SPE A, B och C), "mouse mammary tumor virus proteins" (MMTV), "Streptococcal M proteins", Clostridial perfringens enterotoxin (CPET) m.fl. För en översikt över superantigen och deras egenskaper, se Kotzin et al (Adv. Immunol. 54 (1993) 99-166).

Som funktionellt superantigen har man även betraktat Pseudomonas exotoxin A på grund resultat som indikerar detta toxin kan processas intracellulärt av "accessory cells" till fragment som sedan i sin tur kan uttryckas på cellytan för att där binda till Vβ-kedja med åtföljande aktivering av T-celler (Pseudomonas exotoxin A; Leegard et al., Cell. Immunol. 135 (1991) 372-382).

Superantigen som sådant har föreslagits som terapeutikum vid ett antal olika sjukdomar för att via allmän aktivering av immunsystemet uppnå kurativa effekter (Kalland et al., WO 9104053; Terman et al., WO 9119680; Terman et al., WO 9324136; Newell et al., Proc. Natl. Acad. Sci. USA 88 (1991) 1074-1078).

I samband med vaccin har man även föreslagit att använda superantigen som muterats till att sakna TCR bindand förmåga (Kappler & Marrak, WO 9314634).

Mutation av superantigen har tidigare beskrivits (Kappler & Marrak, WO 9314634; Kappler et al., J. Exp. Med. 175 (1992)

387-96; Grossman et al, J. Immunol. 147 (1991) 3274-81; Hufnagle et al., Infect. Immun 59 (1991) 2126-34).

Vi har tidigare för slaget att använda superantigen kovalent konjugerat med antikropp som terapeutikum för att lysera celler som uppvisar en struktur mot vilken antikroppen är riktad (Dohlsten et al., WO 9201470; Lando et al., Cancer Immunol. Immunother. 36 (1993) 223-8; Kalland et al., Med. Oncol. Tumor Pharmacother. 10 (1993) 37-47; Lando et al J. Immunol 150 (8 part 2) (1993) 114A (Joint Meeting of the American Association of Immunologists and the Clinical Immunology Society, Denver, Colorado, USA May 21-25 (1993)): Lando et al., Proc. Am. Assoc. Cancer Res. Annu. Meet. 33(0) (1992) 339 (Annual Meeting of the American Association for Cancer Research, San Diego, California USA, May 20-23 (1992)); and Dohlsten et al., Proc. Natl. Acad. Sci. USA 88 (1991) 9287-91). Aktuella sjukdomar har varit kancer, virusinfektioner, parasitangrepp, och autoimmuna sjukdomar och andra sjukdomar som är associerade med celler som uttrycker sjudoms-specifika ytstrukturer. Det experimentella arbete som utforts har framst varit inriktat på konjugat innehållande rekombinantframstallt SEA och olika anti-kancer antikroppar. Konjugaten som sådana har uppvisat något reducerad förmåga att binda till MHC klass II antigen jamfört med icke-konjugerad form av det superantigen som ingått i konjugaten. Huruvida en sankt formåga att binda till MHC Klass II antigen är bra eller dåligt for att uppnå optimal lys och terapeutisk effekt har varit oklar.

Immunterapiforsok med SEB kemiskt konjugerat med tumörspecifik anti-idiotyp antikropp har tidigare beskrivits av Ochi et al (J. Immunol. 151 (1993) 3180-3186).

Uppfinningens mål

Ett första mål med uppfinningen är att forbättra tidigare kända superantigen-antikroppskonjugat med avseende på förhållandet mellan generell immunstimulering och riktad cytotox. stimulering resulterar i aktiverade T-lymfocyter och är beroende av superantigenets formåga att binda både till T-cell receptorn och MHC klass II antigen.

Ett andra mål med uppfinningen ar att forbattra selektiviteten av lysen - mellan celler som exponerar antigenet (mot vil-

ket antikroppsdelen av konjugatet är riktat) och andra cell r som exponerar MHC klass II.

Ett tredje mål med uppfinningen är att erbjuda konjugat som kan användas som terapeutikum vid behandling av däggdjur som lider av kancer, autoimmuna sjukdomar, parasitangrepp, virusinfektion eller andra sjukdomar som är associerade med celler som på sin yta uttrycker strukturer som är specifika för respektive sjukdom.

Ett fjärde mål med uppfinningen är att generellt erbjuda konjugat av det aktuella slaget med sänkt affinitet för MHC klass II antigen.

Uppfinningen

Uppfinningens huvudaspekt är ett konjugat som innehåller

a) en biospecific affinitetsmotpart som är riktad mot en struktur till vilken man onskar binda konjugatet och

b) en peptid som

i) är harledd från ett superantigen,

ii) har förmåga att binda till T-cell receptorns Vß-kedja, och

iii) har en modifierad förmåga att binda till MHC klass II antigen jamfort med det superantigen från vilket peptiden är harledd (vildtyp av superantigen = SA(wt)).

Peptiden och affinitetsmotparten är kovalent sammanbundna via en brygga (B).

Uppfinningens foredragna konjugat har förmåga att aktivera och styra T-lymfocyter till selektiv lys av celler som på sin yta uppvisar den struktur mot vilken affinitetsmotparten är riktad. Med detta avses att konjugaten skall förorsaka cytolys i en SADCC-metod (Superantigen Antibody Dependent mediated Cellular Cytotoxicity). Se den experimentella delen nedan och våra tidigare citerade publikationer som avser konjugat mellan superantigen och antikroppar (ex.vis Dohlsten et al., WO 9201470).

Strukturen hos uppfinningens konjugat är analog med tidigar beskrivna superantigen-antikroppskonjugat (Dohlsten et al., WO 9201470 som harmed inkorporeras "by reference"), d.v.s. konjugatet uppfyller formeln

dar T står för den biospecifika affinitetsmotpart n, SA(m) är det modifierade superantig net (peptiden enligt ovan), och B är en kovalent brygga som sammanbinder T och SA(m).

T kan i princip vara vilken som helst struktur som binder via biospecifik affinitet till en motpart men är främst riktad mot en cellytestruktur, företrädesvis sjukdomsspecifika sådana enligt ovan. Den struktur mot vilken T är riktad är vanligtvis skild från (a) det Vβ-epitop, som den superantigenhärledda p p-tiden (SA(m)) binder till, och även (b) från den MHC klass II epitop, som det ursprungliga superantigenet binder till. T är främst valt bland interleukiner (ex.vis interleukin-2), hormoner, antikroppar eller antigenbindande fragment av en antikropp, tillväxtfaktorer, etc. Se t.ex. Woodworth, Preclinical and Clinical Development of Cytokine Toxins" presenterat vid konferensen "Molecular Approaches to Cancer Immunotherapy", Ashville, North Carolina, november 7-11, 1993. För närvarande mindre viktiga exempel på T är polypeptider som binder till konstanta domäner i immunglobuliner (ex.vis proteinerna A, G och L), lektiner, strepavidin, biotin etc.

Vid prioritetsdagen ansågs det föredraget att T var en antikropp eller ett antigenbindande fragment av en antikropp (inkluderande Fab, $F(ab)_2$, Fv, "single chain" antikropp etc), speciellt antikroppsaktiva fragment (såsom Fab) av antikropp riktad mot den s.k. C242 epitopen (Lindholm et al., WO 9301303) eller andra kancerspecifika epitoper.

I det fall T är an antikropp är den främst monoklonal eller en blandning av ett andligt antal monoklonaler (ex.vis 2, 3, 4, 5 eller flera). För icke-terapeutisk användning kan T svara mot en polyklonal antikropp.

T behöver nödvändigtvis inte ha polypeptidstruktur.

SA(m) är främst ett muterat superantigen men kan potentiellt även vara ett kemiskt modifierat superantigen, inkluderande fragment av superantigen med förmåga att binda till Vβ-kedjan i T-cell receptorn. Förmågan hos SA(m) att binda Vβ-k dja är företrädesvis riktad mot en struktur/epitop som är associerad med superantigenaktivering av T-celler.

Med muterat superantigen avses att dess ursprungliga förmåga att binda till MHC klass II antigen har modifierats på gen tisk

nivå så att en eller flera aminosyror ersättes av andra, införes eller avlägsnas i det nativa superantigen t.

SA(m) kan vara uppbyggt av ett antal olika aminosyradelsekvenser, var och en harstammande från olika superantigen.

SA(m) som sådan kan uppvisa sänkt immunogenicitet och toxicitet jamfor med det ursprungliga superantigenet.

Potentiellt kommer andra grupper/substanser som har förmåga att korsreagera med T-cell receptorns Vβ-kedja att kunna utnyttjas ekvivalent med superantigen (SA(m)) som muterats enligt ovan. Sådana grupper/substanser kan vara av icke-polypeptidstruktur.

Såväl T som SA(m) kan vara framstallda med rekombinant teknik.

Bryggan B kan valjas på samma satt som tidigare beskrivits (Dohlsten et al., WO 9201470), d.v.s. den skall främst vara hydrofil och uppvisa en eller flera strukturer valda bland amid, tioéter, eter, disulfid, etc. Finns osubstituerade obrutna kolvatekedjor skall de garna vara korta, ex.vis med mindr an 5 kolatomer i följd och helst sakna aromatiska ringar såsom fenyl. De viktigaste bryggorna B ar de som erhålles i rekombinanta konjugat, d.v.s. då konjugeringen sker på genetisk nivå. Har föredras oligopeptidbryggor vilka framst innehåller hydrofila aminosyrarester, såsom Gln, Ser, Gly, Glu och Arg.

Konjugatet enligt uppfinningen kan innehålla en eller flera modifierade superantigen per biospecifik affinitetsmotpart och vice versa. Detta innebar att T i formeln ovan forutom den biospecifika affinitetsmotparten även kan innehålla en eller flera modifierade superantigen. Analogt gäller för SA(m). I T och SA(m) kan dessutom även finnas andra strukturer. Föredraget ar att antalet modifierade superantigen per affinitetsmotpart ar en eller två.

Syntes av uppfinningens nya konjugat kan utföras enligt i huvudsak två olika principer: 1. Rekombinant och 2. Kemisk sammankoppling av T och SA(m). Metoderna ar för fackmannen valkanda och omfattar ett stort antal varianter. Det följ r att uppfinningen framst avser artificiella konjugat d.v.s. konjugat som ej ar naturligt forekommande.

Vid kemisk sammankoppling mellan ett modifierat superantig n och affinitetsmotparten utnyttjas ofta funktionella grupper

(ex.vis primära aminogrupper eller karboxylgrupper), vilka finns på flera stallen i respektive förening. Detta innebär att den slutliga produkten kommer att innehålla en blandning konjugatmolekyler dar ingående molekyler skiljer sig från varandra med avseende på den position vid vilken sammankoppling skett.

För rekombinant framställda konjugat (fusionsproteiner) gäller att framställd konjugatsubstans blir enhetlig med avseend på position för sammankoppling. Antingen kopplas aminoterminalen i det modifierade superantigenet till karboxyterminalen i affinitetsmotparten, eller vice versa. För Fab-fragment av antikroppar kan antingen latta eller tunga kedjan användas i n sådan fusion. För narvarande ar rekombinanta konjugat att föredra, med foretrade for att aminoterminalen i det modifierade superantigenet ar kopplad till den forsta konstanta domänen av den tunga antikroppskedjan.

Två olika metoder kan anvandas for att ta fram större mängder superantigen (inkluderande muterade och fuserade former) i E. coli: intracellular framstallning eller sekretion. Den senare metoden foredras har, framst av två skal: (i) den medger att superantigenet får en nativ amino-terminal och (ii) korrekt veckat protein kan renas från periplasman och mediet. Intracellular framstallning innebar mer komplicerad rening och betyd r vanligen att proteinet måste (åter)veckas in vitro (för att få en korrekt struktur). Detta utesluter inte att man kan framstalla aktiva konjugat aven i andra vardceller, ex.vis i eukaryota celler som jast eller mammalieceller.

Framstallning av muterade superantigen och selektion av mutanter med modifierad bindningsförmåga (affinitet) till MHC klass II antigen kan ske på kant sätt (ex.vis Kappler et al., J. Exp. Med. 175 (1992) 387-396). Se aven vår experimentella del.

Ett konjugats förmåga att binda till T-cell receptorns Vβ-kedja och till målstrukturen samt att fororsaka lys av en målcell bestams bland annat av peptiden (SA(m)) som är härledd från superantigen, biospecifik affinitetsmotpart (T) och bryggans langd (B) och struktur. Fackmannen kan optimera uppfinningens konjugat med avseende på bindningsförmåga och förmåga att ge lys genom att studera samband mellan effekt och struktur med hjalp av de modeller som utnyttjats i samband med tidigare

kända superantigen-antikroppskonjugat (se ovan angivna litteraturstallen). Se även den experimentella delen nedan.

Med modifierad förmåga att binda till MHC klass II antigen avses främst att kvoten $IC_{50}(SA(wt):IC_{50}(SA(m)$ är < 0,9 (< 90%), såsom < 0,5 (< 50%) och eventuellt även < 0,01 (< 1 %), alternativt att kvoten för affinitetskonstanterna ($K_d(SA(wt):K_d(SA(m))$ uppfyller samma villkor (K_d mätt i nM). För bestämning av $IC_{50}(SA(wt)$, $IC_{50}(SA(m)$, $K_d(SA(wt)$ och $K_d(SA(m))$ se den experimentella delen.

Det är tidigare känt att vissa superantigen kan ha två eller flera säten som binder till MHC klass II antigen (Fraser et al., In: Superantigens: A pathogens view on the immune system. Eds. Huber & Palmer, Current Communications in Cell Molecular Biology 7 (1993) 7-29). För denna typ superantigen gäller att bindningsförmågan skall vara modifierad hos minst ett av säten-na, ex. vis som en reduktion av nyss namnda dignitet. Eventuellt kan det racka med att superantigenet modifierats så att skillnaden i affinitet för två säten som binder MHC klass II antigen ändrats, förslagsvis > 10% och företrädesvis genom reduktion av affiniteten i minst ett säte.

Det modifierade superantigenets (peptiden i icke-konjug rad form) förmåga att binda till Vβ-kedjan i T-cell receptorn bör generellt vara > 0.01 % av motsvarande förmåga för det superantigen från vilket peptiden härstammar, dock gärna > 0,1 % eller > 1 % (mätt på motsvarande sätt som bindningsförmåga till MHC klass II antigen). När peptiden är konjugerad/fuserad till en biospecifik affinitetsmotpart som är riktad mot en ytstruktur på en cell och man önskar lysera cellen, är det väsentligt att bindningsförmågan (affiniteten) är sådan att konjugatet kan rikta och aktivera T-lymfocyter för lys av cellen ifråga (SADCC enligt den experimentella delen med anpassning till det aktuella superantigenet). Detta i sin tur innebär som regel att nyss nämnda villkor för bindningsförmåga till Vβ-kedja uppfylles. För föredragna SA(m) är bindningsförmågan till TCR Vβ ofta omkring 100 % mätt som SADCC.

Main use of the conjugat s/fusion proteins of th inv nti n.

The conjugates according to the invention are primarily intended for the treatment of the same diseases as the

conjugates between normal superantigens and antibodies. S e the above-mentioned publications. Thus they may be administered either as the main therapy or as adjuvant therapy in conn ction with surgery or other drugs.

The pharmaceutical composition of the invention comprises formulations that as such are known within the field but now containing our novel conjugate. Thus the compositions may be in the form of a lyophilized particulate material, a sterile or aseptically produced solution, a tablet, an ampoule etc. Vehicles such as water (preferably buffered to a physiologically pH value by for instance PBS) or other inert solid or liquid material may be present. In general terms the compositions are prepared by the conjugate being mixed with, dissolved in, bound to, or otherwise combined with one or more water-soluble or water-insoluble aqueous or non-aqueous vehicles, if necessary together with suitable additives and adjuvants. It is imperative that the vehicles and conditions shall not adversely affect the activity of the conjugate. Wat r as such is comprised within the expression vehicles.

Normally the conjugates will be sold and administered in predispensed dosages, each one containing an effective amount of the conjugate that, based on the result now presented, is believed to be within the range of 10 µg - 50 mg. The exact dosage varies from case to case and depends on the patient's weight and age, administration route, type of disease, antibody, superantigen, linkage (-B-) et.

The administration routes are those commonly known within the field, i.e. a target cell lysing effective amount or a therapeutically effective amount of a conjugate according to the invention is contacted with the target cells. For the indications specified above this mostly means parenteral administration, such as injection or infusion (subcutaneously, intravenously, intra-arterial, intramuscularly) to a mammal, such as a human being. The conjugate may be administered locally or systemically.

By "target cell lysing effective amount" is contemplat d that the amount is effective in activating and directing T-lymphocytes to destroy the target cell.

Alternativ fi lds of us .

The inventive conjugates can also be employ d to quantitatively or qualitatively detect the structur against which the target-seeking group (T) is directed. In general these methods are well-known to people in the field. Thus, the modified superantigen may function as a marker group within immunoassays and immunohistochemistry meaning that the mark r group in turn is detected by for instance an antibody that is directed towards the peptide (SA(m)) and labelled with an enzyme, isotope, fluorophor or some other marker group known per se. Another method of analysis is to detect in a cell population cells that on their surface express a structure capable of binding to the target-seeking group (T). This use means that a sample from the cell population is incubated with T-lymphocytes together with the present inventive conjugat as in an SADCC assay. In case the incubation leads to cell lysis this is an indication that the population contains cells that on their surface express the structure.

EXPERIMENTAL PART

MANUFACTURE OF RECOMBINANT PROTEINS

Antibodies

The experimental work in connection with the invention has primarily been done with monoclonal antibody C215 as a mod l substance. This antibody is directed against an antigen in th GA-733 family (see for instance EP 376,746) and references cited therein and Larsson et al., Int. J. Canc. 32 (1988) 877-82). The C215 epitope has been judged not to be sufficiently specific for cancer treatment in humans. At the priority dat mab C242 (Lindholm et al., WO 9301303) was believed to be a better candidate, as judged from experiments with its fusion product with wild-type SEA.

Bacterial strains and plasmids

The E. coli strains UL635 (xyl-7, ara-14, $T4^R$, ΔompT) and HB101 (Boyer and Roulland-Dessoix, J. Mol. Biol. 41 (1969) 459-472) were used for the expression and cloning, respectively. The vector pKP889 was used for expression of Fab-SEA fusion proteins (derived from the murine antibody C215) and the vectors pKP943 and pKP1055 for secretion of SEA (Fig 1). Th Fab-SEA expression vector pKP889 is identical to pKP865 (Dohlsten et al, Proc. Natl. Acad. Sci. USA (1994) in press) except that the spacer between C_H1 and SEA is GlyGlyAlaAlaHisTyrGly. Expression from pKP943 yields SEA with the native amino terminus. The use of pKP1055 results in SEA having a Gly residue added at the amino terminus. In both vectors the signals from staphylococcal protein A (Uhlén et al., J. Biol. Chem. 259 (1984) 1695-1702) are used for transcription and translation and a synthetic signal peptide for secretion (L. Abrahmsén, unpublished).

In vitro mutagenesis

Mutations were made by polymerase chain reactions run on a Perkin Elmer Thermocycler. The reaction mixture (100 µl) contained: 1 x PCR buffer from Perkin Elmer-Cetus (10 mM Tris/HCl pH 8.3, 1.5 mM $MgCl_2$, 0.001 % (w/v) gelatine, an additional 2 mM $MgCl_2$, 0.4 mM dNTPs (Perkin Elmer Cetus), 2.5

units of Ampli Taq DNA polymerase (Perkin Elmer Cetus) and 100 ng DNA template. Primers were added to a final conc ntration of 0.8 µM. The original template was a plasmid containing Staphylococcus aureus enterotoxin A gene identical to the one published by Betley et al. (J. Bacteriol. 170 (1988) 34-41), except that the first codon (encoding Ser) was changed to TCC to furnish a Bam HI site at the 5' end of the gene. Later a derivative containing more unique restriction enzyme sites introduced by silent mutations was used. Mutations introduc d next to a restriction site were made with one set of primers, one of these spanning the mutation and the restriction sit . For most mutations two set of primers had to be used and the PCR was performed in two consecutive steps. A new restriction enzyme site was introduced together with each mutation to enable facile identification. Oligonucleotides used as primers were synthesized on a Gene Assembler (Pharmacia Biotech, Sweden). To confirm each mutation the relevant portion of the nucleotide sequence was determined on an Applied Biosystems DNA-Sequenser using their Taq DyeDeoxy Termination Cycle Sequencing Kit.

Protein production and analysis

E. coli cells haboring the different gene constructs were grown overnight at room temperature (Fab-SEA vectors) and at 24-34°C (secretion vectors, the optimum depends on the mutation). The broth was 2 x YT (16 g/l Bacto trypton, 10 g/l Bacto yeast extract, 5 g/l NaCl) supplemented with kanamycin (50 mg/l). Fusion proteins were induced by addition of isopropyl-β-D-thiogalactoside to a final concentration of 100 µM. (The protein A promotor used in the expression of non-fused SEA is constitutive). The cells were pelleted at 5000 x g and the periplasmic contents were released by gently thawing the previously frozen cell pellet in 10 mM Tris-HCl (pH 7.5) on ice during agitation for 1 hour. The periplasmic extracts were clarified by centrifugation at 9500 x g for 15 minutes. The Fab-SEA proteins were used without further purification. SEA and Gly-SEA were further purified by affinity chromatography on an anti-SEA antibody column. Polyclonal rabbit anti-SEA antibodies were previously collected from rabbits preimmunized

with SEA and purified by affinity chromatography on prot in G Sepharose® (Pharmacia Biotech).

Protein Analysis

The proteins were separated in precast polyacrylamide SDS Tris-Glycine Novex gels (gradient 4-20 % or homogenous 12 %, Novex novel experimental technology) and either stained with Coomassie Blue or used in Western blot. Polyclonal rabbit anti-SEA antibodies (above) were used to detect SEA in Western blot analysis, followed by porcine anti-rabbit Ig antibodies, and rabbit anti-horseradish peroxidase antibodies and peroxidase. With Fab-SEA fusion proteins peroxidase conjugated rat antibodies recognizing the kappa chain were also used (AAC 08P, Serotech LTD, England). 3,3'-diaminobenzidine (Sigma) was used for visualization of peroxidase.

Circular dichroism (CD) spectra were collected in a J-720 spectropolarimeter (JASCO, Japan) at room temperature (22-25°C) in 10 mM phosphate buffer, pH 8.2, with 0.02 mM $ZnSO_4$ and 0.005 % (v/v) Tween® 20. The scanning speed was 10 nm/min and each spectrum was averaged from five subsequent scans. The cell path length was 1 mm and the protein concentration 0.2 to 0.5 mg/ml. Guanidine hydrochloride (Gdn-HCl) denaturations at equilibrium were measured at 23°C by CD at 222 nm with a protein concentration of 0.3 mg/ml and a cell path length of 1 mm. These data were used to calculate the apparent fraction of unfolded protein (F_{app}). Equilibrium unfolding parameters were derived by fitting the data to a two-site folding process (Hurle et al., Biochemistry 29 (1990) 4410-4419.

BINDING AND FUNCTIONAL ASSAYS IN VITRO

Materials

Reagents: RPMI 1640 medium obtained from Gibco, Middlesex, England was used. The medium had a pH of 7.4 and contained 2 mM L-glutamine (Gibco, Middlesex, England)), 0.01 M HEPES (Biological Industries, Israel), 1 mM $NaHCO_3$ (Biochrom KG, Germany), 0.1 mg/ml Gentamycin sulphate (Biological Industri s, Israel), 1 mM Na-pyruvate (JRH Biosciences Industries, USA), 0.05 mM mercaptoethanol (Sigma Co., USA), 100 times concentrated non-essential amino acids (Flow Laboratories,

Scotland) and was supplemented with 10 % fetal bovine s rum (Gibco, Middesex, England). Recombinant SEA(wt), SEA(m) and the fusion products C215Fab-SEA(wt) and C215Fab-SEA(m) wer obtained as described above. Human recombinant IL-2 was from Cetus Corp., USA. Mitomycin C was from Sigma Co., USA. $Na_2{}^{51}CrO_4$ was obtained from Merck, Germany. Phosphate buffer d saline (PBS) without magnesium and calcium was received from Imperial, England.

Cells: The human colon carcinoma cell line Colo205 and th B cell lymphoma cell line Raji were obtained from American Type Cell Culture Collection (Rockville, MD) (expressing HLA-DR3/w10, -DP7, -DQw1/w2). The EBV-transformed lymphoblastoid B cell line BSM was a generous gift from Dr van De Griend, Dept of Immunology, Dr Daniel den Hoed Cancer Center, Leiden, the Netherlands. The cells were repeatedly tested for mycoplasma contamination with Gen-Probe Mycoplasma T.C. test, Gen-Probe Inc., San Diego.

SEA activated T cell lines were produced by activation of mononuclear cells from peripheral blood. The blood was receiv d as buffy coats from blood donors at the University Hospital of Lund. The PBMs were stimulated at a concentration of $2x10^6$ cells/ml with mitomycin C treated SEA coated BSM cells (preincubated with 100 ng/ml SEA) in medium with 10 % FCS. The T cell lines were restimu'ated biweekly with 20 U/ml human recombinant IL-2 and weekly with mitomycin C treated SEA coated BSM cells. The cell lines were cultivated for 4-12 weeks before being used in the assay.

The viability of the effector cells, as determined by trypan blue exclusion, exceeded 50 %.

Determination of MHC class II binding characteristics of wild-type and mutant SEA

Radioiodination procedure. Appropriate amounts of wild-type or mutant SEA were radiolabeled with 10 to 25 mCi $Na^{125}I$ using enzymobeads with the lactoperoxidase technique (NEN, Boston, MA). The reaction was stopped by quenching with sodium azide and protein-bound radioactivity was separated from free iodine by filtration through a PD-10 column(Pharmacia Biotech AB, Sollentuna, Sweden) with R10 medium as elution buffer.

Conditions were chosen to obtain a stoichiometric ratio between iodine-125 and protein of ≤ 2:1. The radiochemical purity was verified by size-exclusion chromatography on a TSK SW 3000 HPLC column. The effect of the radioiodination on the binding activity was only tested for wild-type SEA and found not to be affected (data not shown).

Direct binding assay. Raji cells, $6x10^4$/100 µl, previously cultivated in R10 medium, were added to conical polypropylene tubes and incubated (22°C/45 min) in triplicate with 100 µl/tube of serially diluted ^{125}I-labeled wild-type or mutant SEA. The cells were washed with 2 ml 1 % (w/v) bovine serum albumin (BSA) in 10 mM phosphate-buffered saline (PBS), pH 7.4, centrifugated at 300 x g for 5 minutes and aspirated. This procedure was repeated twice. Finally, the cells were analyzed for cell-bound radioactivity in a gamma counter (Packard Instruments Co, Downers Grove, IL). The apparent dissociation constant, K_d, and the number of binding sites, N, at saturation were calculated according to Scatchard (Ann. N.Y. Acad. Sci. 51 (1949) 660-72) after subtraction of non-specific binding (i.e. binding after incubation with R10 medium alone.

Inhibition assay (inhibition of ^{125}I-labeled wild type SEA binding by mutant SEAs). These inhibition experiments were carried out as is described for the direct binding assay with slight modifications. Briefly, 50 µl of ^{125}I-labeled wild type SEA was allowed to compete with an excess of unlabeled wild type or mutant SEA (50 µl/tube) for binding to $6x10^4$/100 µl Raji cells. A tracer concentration yielding ≈ 40 % bound radioactivity in the direct assay was used to obtain maximal sensitivity in the inhibition assay. The displacement capacity of the competitor was expressed as the concentration yielding 50 % inhibition (IC_{50}) of bound radioactivity. The binding affinity of the mutants relative to wild-type SEA was calculated using the equation:

$$IC_{50}(SEA(wt)) : IC_{50}(SEA(m))$$

In order to analyze whether the mutants compete for binding to the same site on Raji cells as wild type SEA, the binding data obtained with SEA mutants were plotted as a log-logit function and tested for parallelism with the corresponding data for wild type SEA.

Inhibition assay (inhibition of the binding of fluor scent-labeled wild type SEA by unlabeled SEA and SEA mutants). Raji cells (2.5 x 10^5) w re incubated with inhibitor (wild type or mutant SEA; 0-6000 nM) diluted in 50 µl CO_2-independent medium (Gibco) supplemented with 10 % FCS, glutamine and gentamycin at 37°C for 30 minutes. Fluorescein conjugated SEA was added to a final concentration of 30 nM and the samples were incubated for an additional half hour at 37°C. The samples were washed thre times with ice cold PBS supplemented with 1 % BSA (PBS-BSA) and finally kept in 0.4 ml PBS-BSA on ice until they were analyzed. From each sample 10 000 live cells were analyzed for green fluorescence on a FACStar® (Becton Dickinson) flow cytometer and the mean fluorescence value was calculated using the LYSIS II program.

SDCC and SADCC assays of SEA(wt), SEA(m) and their fusion proteins with C215Fab.

SDCC-assays. The cytotoxicity of SEA(wt), SEA(m) and their fusions with C215Fab against MHC class II^+ Raji cells was analyzed in a standard 4 hour $^{51}Cr^{3+}$-release assay, using in vitro stimulated SEA specific T cell lines as effector cells. Briefly, ^{51}Cr labeled Raji cells were incubated at 2.5 x 10^3 cells per 0.2 ml medium (RPMI, 10 % FCS) in microtitre wells at defined effector to target cell ratio in the presence or absence (control) of the additives. Percent specific cytotoxicity was calculated as 100 x ([cpm experimental release - cpm background release]/[cpm total release - cpm background release]). The effector to target cell ratio was 30:1 for unfused SEAs and 40:1 for fusion proteins.

SADCC against of human colon cancer cells. The cytotoxicity of C215-FabSEA(wt), C215-FabSEA(m), SEA and SEA mutants against $C215^+$ MHC class II^- colon carcinoma cells SW 620 was analyzed in a standard 4 hour $^{51}Cr^{3+}$-release assay, using in vitro stimulated SEA specific T cell lines as effector cells. Briefly, $^{51}Cr^{3+}$-labeled SW 620 cells were incubated at 2.5 x 10^3 cells per 0.2 ml medium (RPMI, 10 % FCS) in microtitre wells at effector to target cell ratio 30:1 in the presence or absence (control) of the additives. Percent specific cytotoxicity was calculated as for SDCC assays.

IN VIVO FUNCTIONAL EXPERIMENTS

Tumor c lls. B16-F10 melanoma cells transfected with a cDNA encoding the human tumor associated antigen C215 (B16-C215) (Dohlsten et al., Monoclonal antibody-superantigen fusion proteins: Tumor specific agents for T cell based tumor therapy; Proc. Natl. Acad. Sci. USA, In press, 1994), were grown as adherent cells to subconfluency. The culture medium consisted of RPMI 1640 (GIBCO, Middlesex, UK) supplemented with $5x10^{-5}$ β-mercaptoethanol (Sigma, St Louis, MO), 2 mM L-glutamine (GIBCO), 0.01 M Hepes (Biological Industries, Israel) and 10 % fetal calf serum (GIBCO). The cells were detached by a bri f incubation in 0.02 % EDTA and suspended in ice cold phosphate buffered saline with 1 % syngeneic mouse serum (vehicle) to $4x10^5$ cells/ml.

Animals and animal treatment. All mice were 12-19 weeks old C57Bl/6 mice transgeneic for a T cell receptor Vβ3 chain (Dohlsten et al., Immunology 79 (1993) 520-527). One hundred thousand B16-C215 tumor cells were injected i.v. in the tail vein in 0.2 ml vehicle. On day 1, 2 and 3, the mice were given i.v. injections of 215Fab-SEA(wt) or C215Fab-SEA(D227A) in 0.2 ml vehicles at doses indicated in the figures 5a and 5b. Control mice were given vehicle according to the same schedule. On day 21 after tumor cell injection, the mice were killed by cervical dislocation, the lungs removed, fixed in Bouins solution and the number of lung metastases counted.

RESULTS

"Alanine scanning" of staphylococcal enterotoxin A.

Initially the structure of SEA was unknown and only speculations could be done about what side chains were surface accessible. Therefore, the majority of the mutants were chosen from alignments of homologous superantigens (Marrack and Kappler, Science 248 (1990) 705-711). Conserved (mainly polar) residues were chosen on the rational that some of these superantigens are expected to bind to HLA-DR in a conserved fashion (Chitagumpala et al., J. Immunol. 147 (1991) 3876-3881). Alanine replacements were used according to published strategies (Cunnningham and Wells, Science 244 (1988) 1081-

1085). During the course of this work the available information increased: i) it was shown that a Zn^{2+} ion is important for the interaction between SEA and MHC class II (HLA-DR) (Fras r et al., Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511), ii) a mutational analysis of staphylococcal enterotoxin B (SEB) was presented (Kappler et al., J. Exp. Med. 175 (1992) 387-396), and iii) the structure of SEB was presented (Swaminathan et al., Nature 359 (1992) 801-806).

Our first mutant showing a severely reduced affinity for HLA-DR, Asp227Ala, was found to co-ordinate the Zn^{2+} ion very poorly (data not shown). Assuming a common fold for SEA and SEB, the new data suggested two MHC class II binding regions; one involving the Zn^{2+} ion and one corresponding to the sit defined in SEB. A second set of mutations were made on these assumptions. This second set of mutants were expressed in the form of SEA carrying a glycine added at the amino terminus. First the extension was shown to have no effects on the binding properties of wild type SEA (next section).

Most of the mutants were expressed and secreted by E. coli in a functional form as judged by analysis of the binding of monoclonal antibodies (Table I). Very low amounts were obtained of the mutants E154A/D156A and R160A. Consequently these were excluded from the study. The mutants having an Ala substitution in residues 128, 187, 225 or 227 were not recognized by the monoclonal antibody 1E. The latter two mutants showed a reduced level of expression (more pronounced at 34°C than at 24°C) and migrated faster during SDS PAGE, under denaturing but not reducing conditions (all other mutants migrated as wt SEA, not shown). As judged by CD spectra analysis the structure of D227A could differ slightly from native SEA (figure 2), but the stability was very close to wild-type SEA (measured as resistance towards Guanidine hydrochloride denaturation). The calculated $\Delta\Delta G$ between the mutant and native SEA was -0.16 kcal/mol and is only about 4 % of the $\Delta G°$ values (data not shown). Overall the signals in the CD analysis were low, as expected from a mostly β-sheet structur . It was recently reported that His 225 co-ordinates Zn^{2+} (unpublished data in Fraser et al (Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511). Since Asp 227 is involved in Zn^{2+} co-ordination (above) and

presumably located in the same β-sheet as His 225 this suggests that these two residues constitutes the zinc-binding nucleus found in zinc-co-ordinating proteins (Vallee and Auld, Biochemistry 29 (1990) 5647-5659).

Binding to MHC class II and T cell receptor

The MHC class II affinity was calculated from the amounts needed to compete with fluorescein-labeled wild-type SEA for Raji cell exposing large amounts of MHC class II. The displacement capacity of a mutant was calculated from the concentration yielding 50 % inhibition (IC_{50}) of bound fluorescence compared with the concentration needed with wild-type SEA as the competitor. For wild-type SEA and for some mutants, the result from this analysis was compared with the result from an analysis where ^{125}I labeled wild-type SEA was used as the tracer. As may be seen in Table II, the values obtained from these analyses correlates well

For six selected mutants the binding to MHC class II was measured directly using ^{125}I labeled mutant SEA (Table II). With the mutant His50Ala the values obtained from the two analyses correlated well but with the mutant Phe47Ala a large discrepancy was found: the direct binding indicated only 7 times weaker binding than wild type SEA but both competition analyses demonstrated around 70 times reduced binding. The data from two of the other mutants indicated two separate binding interactions. For the mutants H225A and D227A the affinity was below the detection limit also in this analysis.

We previously showed that fusion proteins composed of the Fab fragment of a carcinoma reactive antibody and SEA could be used to direct cytotoxic T cells to specifically lyse cancer cells, while the interaction between SEA and the T cell receptor (TCR) was too weak to be detected by itself (Dohlsten et al., Proc. Natl. Acad. Sci. USA, in press). Thus, in contrast to analyses involving the isolated superantigen the Fab fusion context enables a functional assay for the interaction between SEA and the TCR, independent of the MHC class II binding. Consequently, the efficiency of the different conjugates to direct T cells to lyse cells recognized by the Fab moiety was monitored in a chromium release assay. This

analysis confirmed that the mutations shown to aff ct the MHC class II binding did not affect the TCR binding (Tabl II).

Biological effects of the mutations

The proliferative effect was measured as the ability to stimulate peripheral lymphocytes to divide. All three mutants that competes very poorly for MHC class II induced little or no proliferation and the intermediate mutant His187Ala display d some proliferative capacity, whereas the other investigated mutants were indistinguishable from the wild type (table III). Harris et al (Infect. Immun. 61 (1993) 3175-3183) recently reported a similar severe reduction in T cell stimulatory activity for the SEA mutants F47G and L48G. Clearly a strong reduction in any of the two suggested binding regions results in a severe effect on the ability to induce proliferation. This suggests that SEA cross-links two molecules of MHC class II leading to dimerization of the TCR and that this is needed to yield a signal transduction.

In contrast the efficiency of the different mutants in directing in vitro stimulated SEA T cells to lyse MHC class II bearing target cells shows correlation with the binding affinity, rather than to the ability to compete (Table III). For example, the efficiency of F47A and D227A are only reduced 2.5 times and 300 times, respectively. Thus, here no inher nt requirement for divalency too is obvious. The increase in multivalency resulting from the significantly larger number of TCRs on the surface of activated T cells might partially shield the effect of a lower avidity in the SEA/MHC class II interaction. That dimerization is not needed to direct T cell cytotoxicity has previously been demonstrated by the use of carcinoma secific bifunctional antibodies containing one anti-CD3 moiety and one anti-carcinoma moiety (Renner et al., Science 264 (1994) 833-35).

In vivo functional xperim nts: The results are represented in figures 6a and 6b. Treatment of mice with C215Fab-SEA(wt) and C215Fab-SEA(D227A) were both highly eff ctive in reducing the number of lung metastases of B16-C215 melanoma cells. The therapeutic effect was essentially identical for he two

variants of the targeted superantigens. Treatment with C215Fab-SEA(wt) resulted in 70 % lethality at dos s of 5 µg/inj ction. In contrast, no mice died when the same dose of C215Fab-SEA(D227A) were used. Taken together, SEA(D227A) is an example of a mutant with reduced toxicity and retained therapeutic efficiency when incorporated in a Fab-SEA fusion protein.

DISCUSSION

The structure of the complex between SEB and HLA-DR was recently reported (Jardetzky et al., Nature 368 (1994) 711-718). Most of the SEB residues identified to be involved in this interaction are conserved in SEA. Our data on mutant D227A indicates a weak affinity for the interaction between this site of SEA (the amino proximal site) and the MHC class II, having a K_d value higher than 8 µM. The K_d for the interaction between SEB and HLA-DR was recently reported to be 1.7 µM (Seth et al., Nature 369 (1994) 324-27). Here the different interactions between SEB, TCR and HLA-DR were investigated and it was shown that the complex between SEB and HLA-DR was not stably maintained in the absence of TCR. Plasmon resonance experiments indicated that this was because of a very fast off-rate. The avidity effects obtained if SEA cross-links two molecules of MHC class II followed by a subsequent dimerization of the TCR could explain how SEA may induce proliferative effects at concentrations well below the K_d. Assuming that the mutation F47A reduces the affinity of the amino proximal site below significance, the K_d of the Zn^{2+} site is around 95 nM. This hypothesis was recently strengthened by the observation that the mutants F47R, F47R/H50A and F47R/L48A/H50D show identical affinity for MHC class II at F47A (unpublished).

Based on the SEB structure (Kappler et al., J. Exp. Med. 175 (1992) 387-396) and on homology alignments (Marrack and Kappler, Science 248 (1990) 705-711), it is strongly suggested that His225 and Asp227 are located in the same β-sheet and thus the side chains could be proximal. Thus, most likely these two residues constitute the zinc-binding nucleus found in zinc-co-ordinating proteins (Vallee and Auld, Biochemistry 29 (1990) 5647-5659). Similarly to these mutants, the mutants with a replacement at residue 128 or 187 are also recognized by all

monoclonals except 1E. Fraser et al (Proc. Natl. Acad. Sci. USA 89 (1991) 5507-5511) showed that Zn^{2+} is bound to SEA and is needed for a high affinity interaction with MHC class II. The affinity for zinc was not affected by the addition of HLA-DR. Based on this observation and the high affinity for Zn^{2+} (K_d of around 1 μM) a co-ordination exclusively provided by SEA and involving 4 fold co-ordination was suggested. Our data indicates an involvement of the four residues N128, H187, H225 and D227. The function of the former two residues is not yet clear; instead of providing a ligand N128 could help in the deprotonation of D227. One argument for this is that the effect of replacing D227 is more severe that when replacing H225.

It was previously reported that there is a lack of correlation between the affinity of different superantigens for the MHC class II and the capacity to stimulate T cells to proliferate (Chintagumpala et al., J. Immunol. 147 (1991) 3876-3881). These results might partly be explained by different affinities of the superantigens towards different TCR Vβ-chains. Here we have observed the same lack of correlation but in contrast to separate superantigens the mutants display identical TCR affinity as shown in the Fab-SEA context. The most likely explanation for the lack of correlation is that two binding regions identified in this analysis represent two separate binding sites that yields not only a co-operative binding, but which results in the cross-linking of two molecules of MHC class II, which in turn yields dimerization of two molecules of the T cell receptor. This would imply that the affinity of both sites are important to obtain the proliferative effect. A high avidity results from the interactions within a hexameric complex involving two molecules of SEA, TCR and MHC class II. Thus the strong affinity/avidity of SEA towards MHC class II enables SEA interaction with the TCR despite a low direct affinity.

Other biospecific affinity counterparts: A fusion protein of SEA(D227A) and an IgG-binding domain of staphylococcal protein A has been produced by recombinant technology and expressed in E. coli. This reagent has successfully been used to target T-lymphocytes to Mot 4 and CCRF-CEM cells (obtained from ATCC)

that are CD7 and CD38 positiv but HLA-DP, -DQ and -DR negative. The Mot 4 and CCRF-CEM c lls wer preincubated with anti-CD7 or anti-CD38 mouse monoclonals (Dianova, Hamburg, Germany). In order to enhance binding between the mous monoclonals and the IgG-binding part of the fusion protein rabbit anti-mouse Ig antibody was also added. In comparison with protein A-SEA(wt), protein A-SEA(D227A) had a deccreased ability to bind to Daudi cells expressing MHC class II antigen.

Table I

Confirmation of mutant structural integrity. The binding of six monoclonal antibodies was monitored.

Mutation	Monoclonal antibody[1]					
	1A	2A	3A	1E	4E	EC-A1
Wild type	+	+	+	+	+	+
D11A/K14A	+	+	+	+	+	+
D45A	+	+	+	+	+	+
F47A	+	+	+	+	+	+
H50A	(+)	+	(+)	+	+	+
K55A	+	+	+	+	+	+
H114A	+	+	+	+	+	+
K123A/D132G	+	+	+	+	+	+
N128A	+	+	+	−	+	+
K147A/K148A	+	+	+	+	−	+
E154A/D156A	ND	ND	ND	+	ND	ND
R160A	ND	ND	ND	+	ND	ND
H187A	+	+	+	−	+	+
E191A/N195A	+	+	+	+	+	+
D197A	+	+	+	+	+	+
H225A	+	+	+	−	+	+
D227A	+	+	+	−	+	+

Footnotes: 1) A plus sign indicates binding, parenthesis indicate 50 to 90 % binding compared with wild type SEA. ND means not determined.

Tabl II

Binding of SEA mutants to the MHC class II and th T cell receptor. The latter was monitored as the ability to dir ct activated cytotoxic T-cells specifically to lyse carcinoma cells using Fab-SEA fusions of the different mutants (SADCC).

Mutation	IC_{50}(nM) SEA-FITC[1]	IC_{50}(nM) ^{125}I-SEA[1]	K_d(nM) ^{125}I labeled[1]	SADCC(% of wild-type[1]
wild type	50	38	13	100[2]
Gly-SEA	50	ND	ND	100[2]
D11A/K14A	50	ND	ND	ND
D45A	53	ND	ND	ND
F47A	3150	2943	95	100
H50A	150	132	32	100
K55A	44	ND	ND	ND
H114A	48	ND	ND	ND
K123A/D132G	188	75	12/237	100
N128A	1150	ND	2.9/76	100
K147A/K148A	58	ND	ND	ND
H187A	1030	602	97	100
E191A/N195A	51	ND	ND	ND
D197A	78	ND	ND	ND
H225A	>9000	9600	ND	ND
D227A	>9000	>10000	>8000	100

Footnotes: 1) ND means not determined. 2) In the Fab-SEA context the spacer between C_H1 and SEA ends with a Gly.

Table III

Biological effects of the mutations. The ability to stimulat resting T cells to proliferate and the ability to dir ct cytotoxic cells to lyse MHC class II exposing target cell w r monitored (SDCC = Superantigen Dependent mediated Cellular Cytotoxicity).

Mutation	Proliferation[1] %	SDCC EC_{50} (relative)[1]
wild type	100	1
Gly-SEA	ND	1
D11A/K14A	ND	0.8
D45A	50	1.3
F47A	<0.2	2.5
H50A	20	1.4
K55A	100	1.3
H114A	ND	1
K123A/D132G	40	2.1
N128A	40	1.2
K147A/K148A	ND	0.7
E154A/D156A	ND	ND
R160A	ND	ND
H187A	15	4
E191A/N195A	100	1.1
D197A	ND	1.3
H225A	<0.2	$3x10^2$
D227A	<0.01	$3x10^2$

Footnotes: ND not determined.

LEGENDS TO THE FIGURES

General: The mutant SEA(D227A) (SEA mutant 9 (= SEA(m9)) was at the priority date the most promising SEA variant. Figures 3-6 therefore presents in vitro and in vivo results with this variant.

Figur 1.

Schematic outline of th plasmids us d to express SEA and C215Fab-SEA. The coding regions and th two transcription terminators following the product genes are indicated by boxes. The gene encoding the kanamycin resistance protein is labeled

Km. lacI is the lac repressor gene. V_H and C_H1 indicates the gene encoding the F_d fragment of the heavy chain of the murine antibody C215. Likewise V_K and C_K indicates the gene encoding the kappa chain. Rop is the gene encoding the replication control protein from pBR322. The promotors directing transcription of product genes are shown as arrows, in pKP889 the trc promotor and in the other two vectors the promotor from staphylococcal protein A (spa). The region containing the origin of replication is indicated by ori. The only difference between SEA encoded by pKP943 and pKP1055 is a glycine residu added at the N-terminus of the latter. The SEA gene contained in the latter vector also contains more unique restriction enzyme sites, introduced by silent mutations.

Figure 2

Circular dichroism spectra for wild type SEA and for the mutants F47A and D227A, representing the most severely reduced mutations in each MHC class II binding region. The solid line is the curve for wild type SEA. The curves for the mutants are dotted or center, F47A respectively D227A.

Figure 3 shows the concentration dependency of superantigen dependent mediated cellular cytotoxicity (SDCC) for SEA(wt) and SEA(m9).

Figure 4 shows the concentration dependency of superantigen dependent cell mediated cytotoxicity (SDCC) for C215Fab-SEA(wt) and C215Fab-SEA(m9).

Figure 5 shows the concentration dependency of superantigen mAb dependent cell mediated cytotoxicity (SADCC) for C215Fab-SEA(wt) and C215Fab-SEA(m9) compared to free SEA(wt).

Figure 6a compares the therapeutic effects obtained in C57B1/6 mice carrying lung metastases of B16-C215 melanoma cells by treatment with C215Fab-SEA(wt) and C215Fab-SEA(m9).

Figure 6b shows toxicity of C215-SEA(wt) and C215-SEA(m9) for the treatments represented in figure 6a.

PATENTKRAV

1. Konjugat innehållande
   a) en biospecific affinitetsmotpart (målsökande grupp) som binder till en förutbestämd struktur och
   b) en peptid som
      (i) innehåller en aminosyrasekvens som är härledd från ett superantigen,
      (ii) har förmåga att binda till en Vβ-kedja på en T-cell receptor, och
      (iii) har en modifierad förmåga att binda till MHC klass II jämfört med det superantigen från vilket peptiden är härledd.

   vilka delar är kovalent sammanbundna.

2. Konjugat enligt krav 1, kännetecknat av att
   a) den biospecifika affinitetsmotparten är riktad mot n ytstruktur på en cell, och att
   b) konjugatet har förmåga att aktivera T-lymfocyter för lys av celler som uppvisar strukturen på sin yta.

3. Konjugat enligt något av kraven 1-2, **kännetecknat** av att den biospecifika motparten är en antikropp eller ett antigenbindande fragment av en antikropp.

4. Konjugat enligt något av kraven 1-3, **kännetecknat** av att det är ett fusionsprotein.

5. Konjugat enligt något av kraven 1-4, **kännetecknat** av att peptiden är ett muterat superantigen.

6. Konjugat enligt något av kraven 1-5, **kännetecknat** av att peptiden är härledd från ett superantigen och att dess förmåga att binda till MHC klass II antigen är förändrad med minst 10 %.

7. Konjugat enligt något av kraven 1-6, **kännetecknat** av att superantigenet är stafylokockenterotoxin A, B, C_1, C_2, D eller E.

8. Konjugat enligt något av kraven 1-7, **kännetecknat** av att strukturen mot vilket den biospecifika affinitetsmotparten är riktad är en struktur som uttrycks på cellytan i förhöjd mängd vid sjukdom, exempelvis vid kancer, virusinfektion, autoimmun sjukdom eller parasitangrepp.

9. Satt att lysera celler från ett däggdjur, **kännetecknat** av att cellerna inkuberas med T-lymfocyter och ett konjugat enligt något av kraven 2-8, i vilket den biospecifika affinitetsmotparten är riktad mot en ytstruktur på cellerna som skall lyseras, under betingelser sådana att cellerna lyseras.

10. Satt att selektivt lysera celler (I) som förekommer tillsammans med andra celler (II) och som uppvisar en ytstruktur som framst förekommer på de celler (I) som skall lyseras, **kännetecknat** av att cellerna (I tillsammans med II) samtidigt kontaktas med ett konjugat enligt något av kraven 2-8, i vilket den biospecifika affinitetsmotparten är riktad mot en ytstruktur på cellerna (I) som skall lyseras, under betingelser sådana att cellerna (I) lyseras.

11. Satt enligt krav 10, **kännetecknat** av att cellerna (I) är associerade med sjukliga tillstånd såsom kancer, virusinfektion, parasitangrepp, autoimmuna sjukdomar etc.

12. Forfarande for att behandla ett sjukligt tillstånd hos daggdjur, vilket tillstånd innebar förekomst av specifika celler som är associerade med tillståndet genom att de uttrycker en sjukdomsspecifik ytstruktur, **kännetecknat** av att man till daggdjuret administrerar en terapeutiskt effektiv mängd av ett konjugat enligt något av kraven 2-8 i vilket konjugat den biospecifika affinitetsmotparten är riktad mot den sjukdomsspecifika ytstrukturen.

SAMMANDRAG

Konjugat innehållande

a) en biospecific affinit tsmotpart (målsokand grupp) som binder till en förutbestämd struktur och

b) en peptid som

(i) innehåller en aminosyrasekvens som är härledd från ett superantigen,

(ii) har förmåga att binda till en Vβ-kedja på en T-cell receptor, och

(iii) har en modifierad förmåga att binda till MHC klass II jamfort med det superantigen från vilket p ptiden är harledd.

vilka delar ar kovalent sammanbundna.

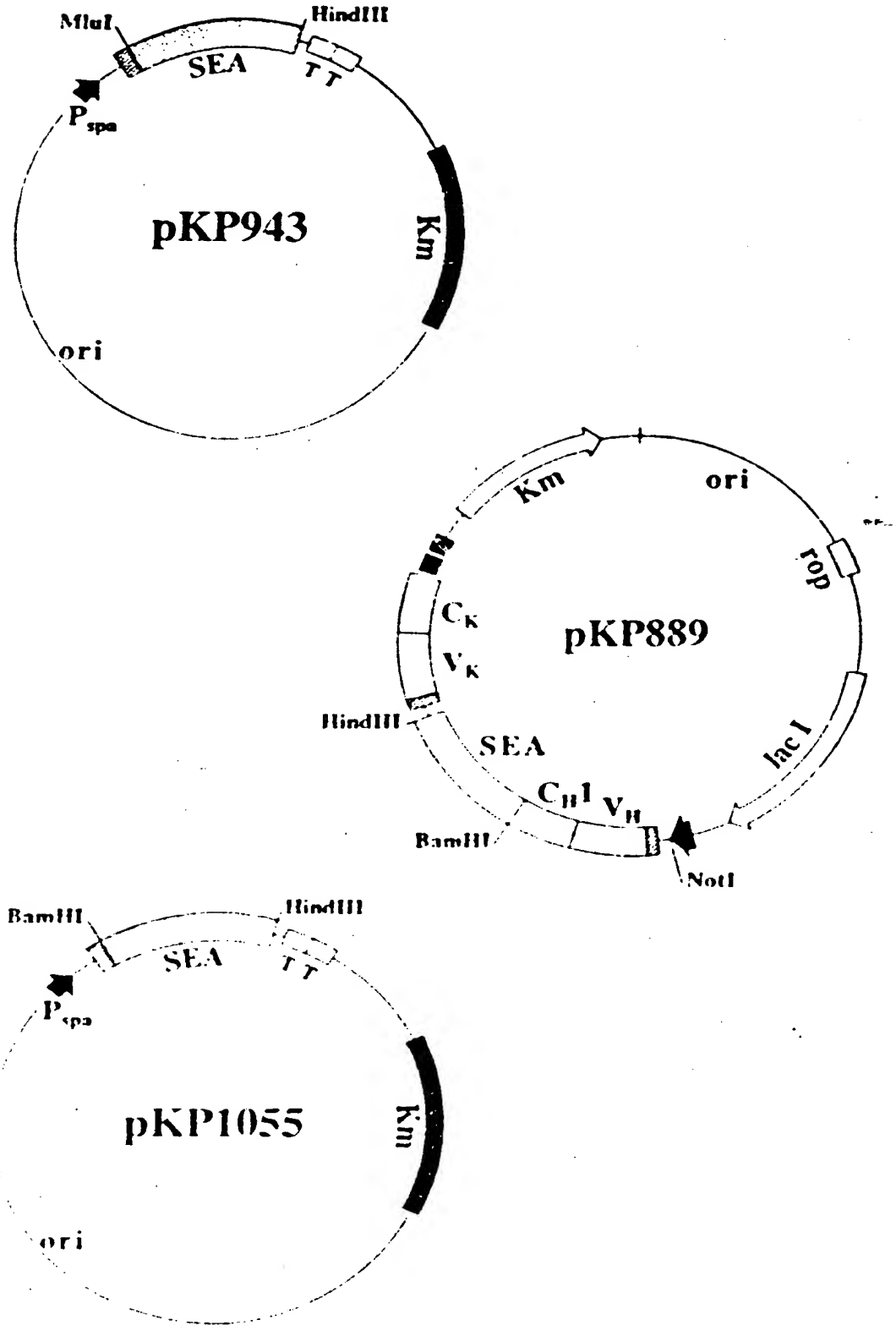
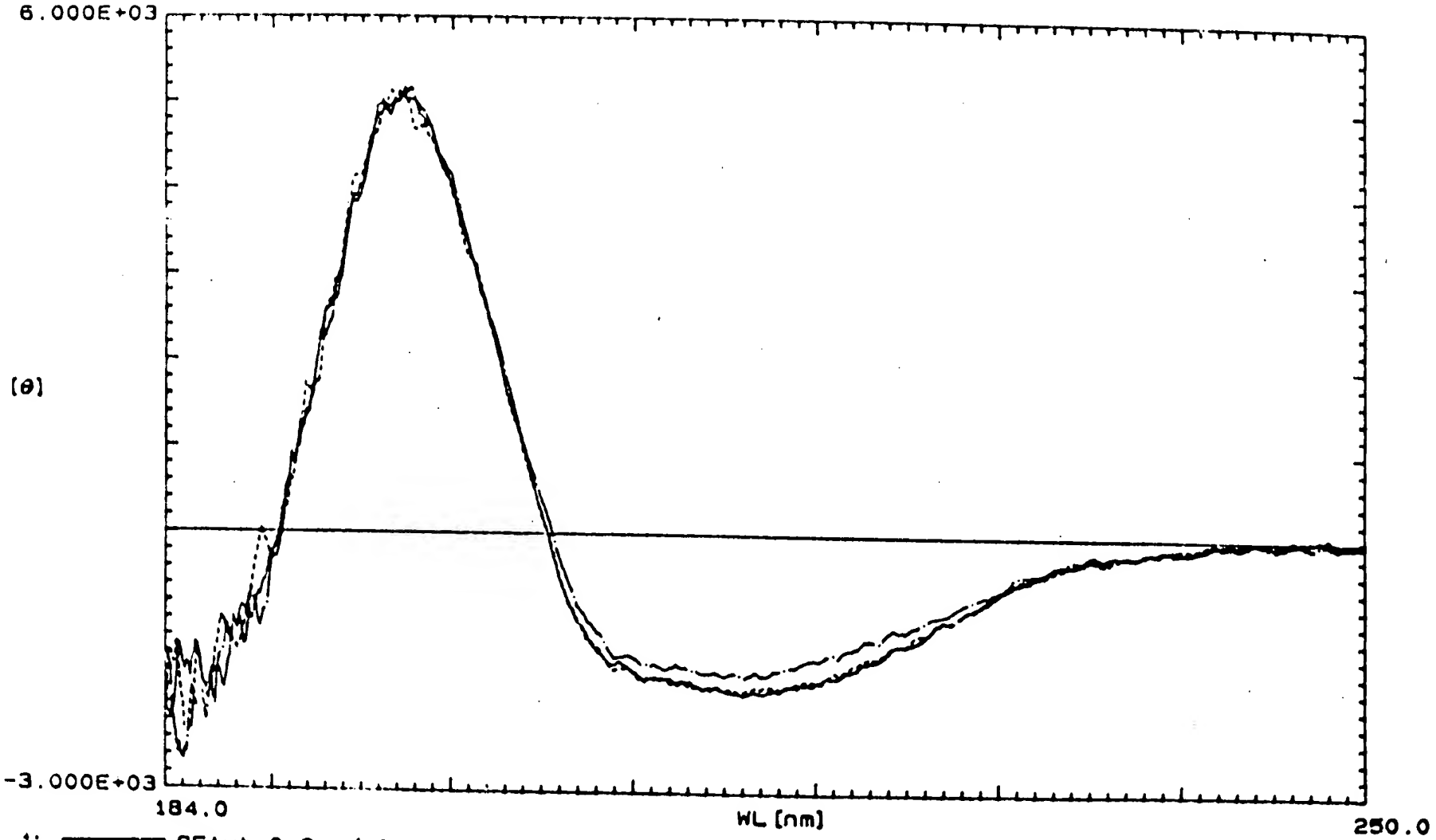


FIGURE 1

FIGURE 2



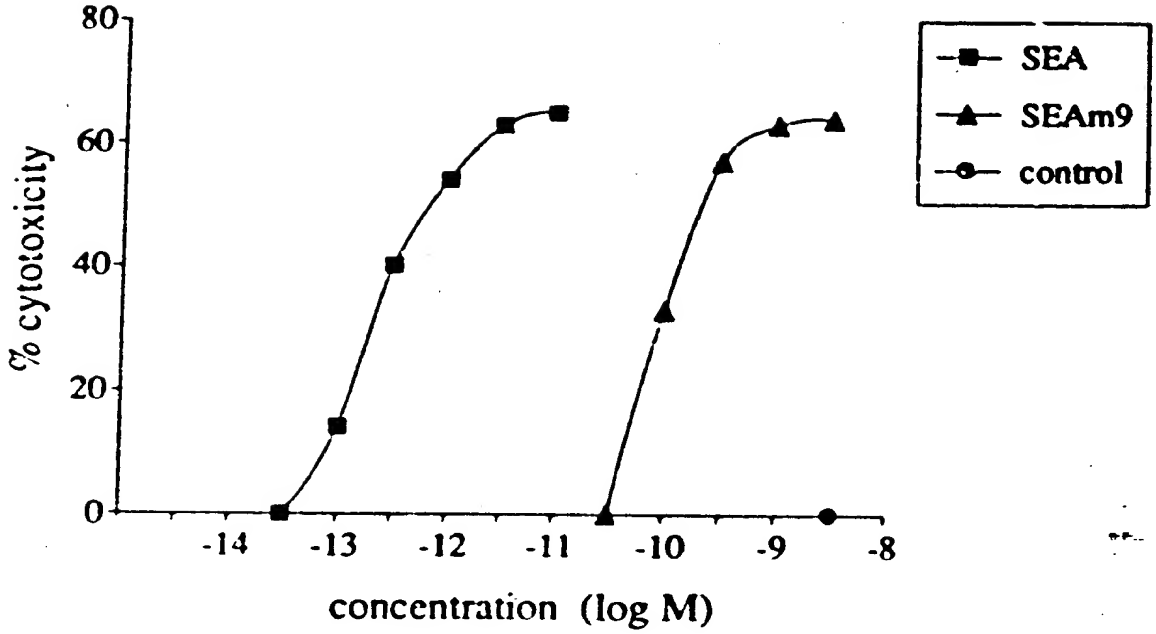

80
60
40
20
0
% cytotoxicity
-14
-13
-12
-11
-10
-9
-8
concentration (log M)
SEA
SEAm9
control

FIGURE 3

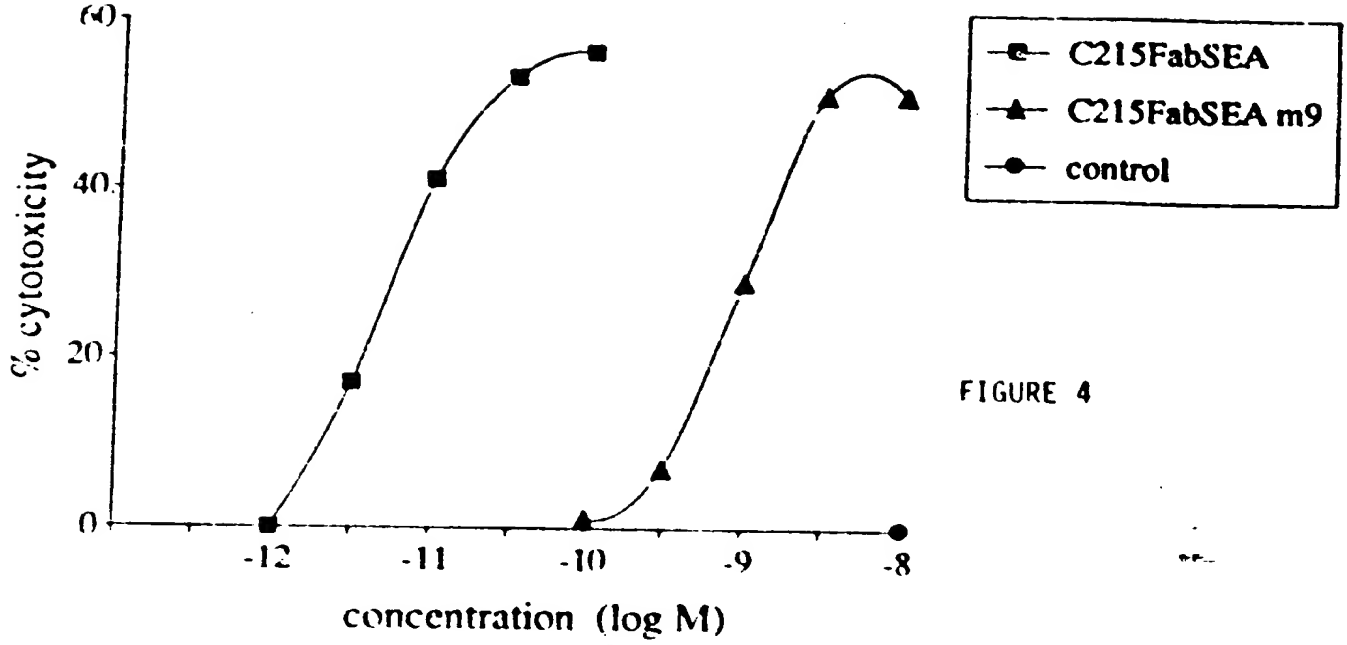


FIGURE 4

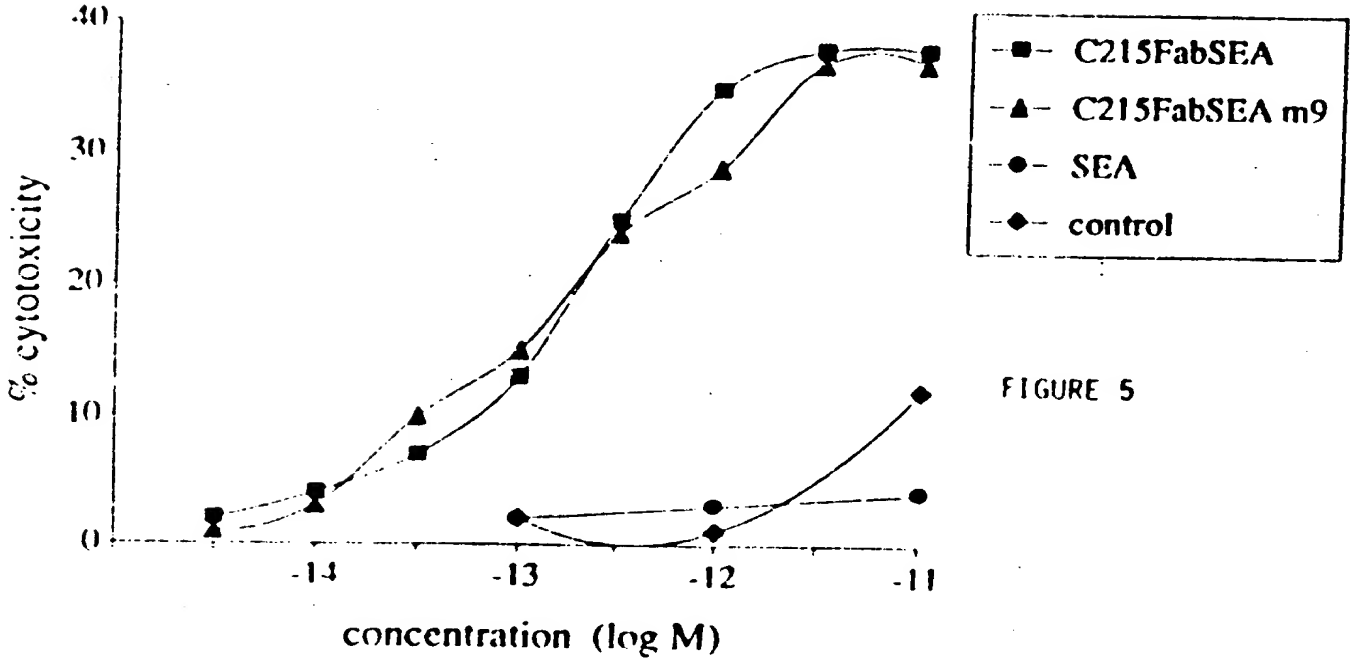


FIGURE 5

Therapeutic effect

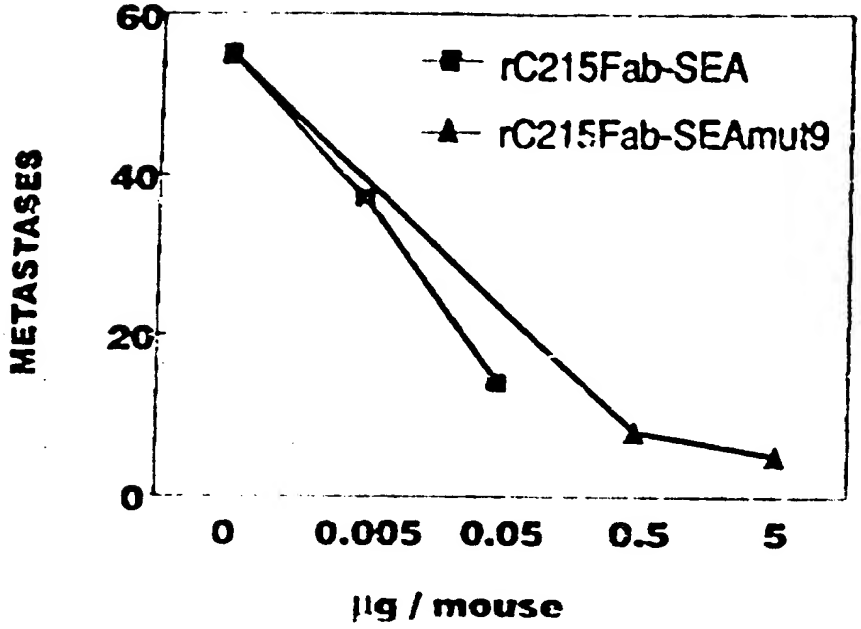


FIGURE 6A

Toxicity

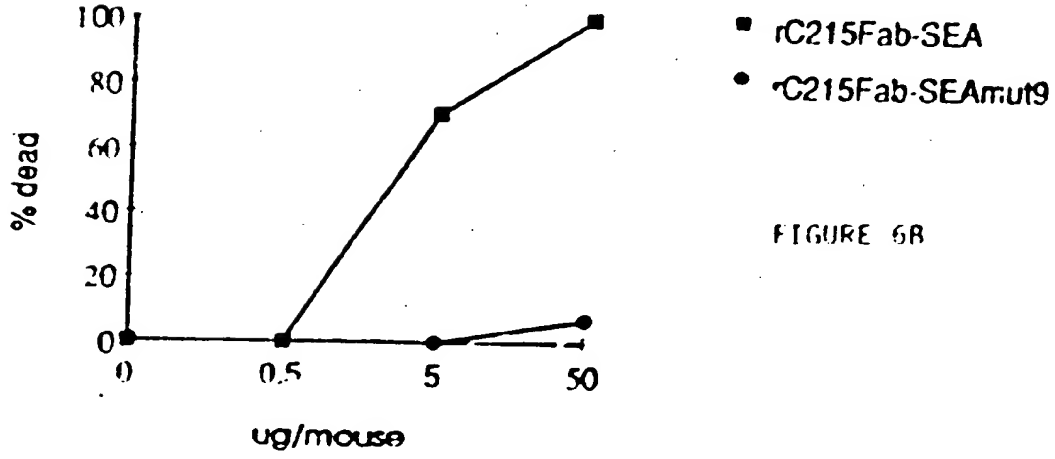


FIGURE 6B